Antioxidant Agents in Alzheimer's Disease

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Abstract: Understanding the molecular mechanisms of neurodegeneration represents a scientific priority as it will allow scientists to more specifically target and simultaneously interrupt the multiple pathologic mechanisms that contribute to the progression of dementia in Alzheimer's disease (AD). Oxidative stress represents one of the key processes in AD pathogenesis, related to formation of both amyloid plaques and neurofibrillary tangles as well as to alteration of many biomolecules. For this reason several antioxidant molecules have been tested in *in vitro* and *in vivo* studies in order to detect more efficacious treatments. Dietary antioxidants seem also to have an important role in AD prevention, as shown by several epidemiological studies. Ongoing clinical trials to assess whether antioxidant supplementation has a role in primary prevention of AD or in delaying the progression of disease in individuals with Mild Cognitive Impairment (MCI), are in progress or planned. Thus, research involving new antioxidants and their potential clinical applications will provide new insights into the molecular basis of neuroprotective mechanisms that may be relevant to AD and other age-related neurodegenerative disorders.

Keywords: Alzheimer's disease, antioxidant, diet, mild cognitive impairment, oxidative stress, treatment.

1. INTRODUCTION

Alzheimer's disease (AD) represents the principal cause of dementia in the elderly. Aging is a major risk factor for AD and up to 40% of the population over 65 years of age might be affected [1]. As people live longer, AD is becoming a major medical and social concern, as over 13 million people with AD are expected in 2050 in the US [2] and the number of new dementia cases per year in the EU will increase from about 1.9 million in the year 2000 to about 4.1 million in the year 2050 [3].

The clinical manifestations of AD include memory dysfunction, which is usually the first symptom, loss of lexical access, spatial and temporal disorientation, impairment of judgment, and often behavioural disturbances. The hallmarks of AD are senile plaques, formed by extracellular deposition of β-amyloid (Aβ) protein, and neurofibrillary tangles (NFT), composed of intracellular precipitation of hyperphosphorylated tau protein. AD is also histopathologically characterized by synaptic loss and nerve cell loss, mainly in the cerebral cortex, in the hippocampus and in the amygdala. A curative treatment for AD is not available currently, which renders the understanding of AD pathophysiology and the search for new treatments urgent needs.

To date, the mechanism or mechanisms responsible for AD remain obscure, despite numerous hypotheses that have been presented. Among these, the oxidative stress hypothesis is appealing because it involves several other hypotheses, including the trace element hypothesis, the mitochondrial bioenergetic decline hypothesis and the Aβ peptide hypothesis [4, 5].

There are indeed several proofs produced in the past decades confirming the pathophysiological role of oxidative damage in AD. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are products of normal cellular aerobic metabolism; they play a dual role as beneficial but also as deleterious species, according to their concentration. Useful effects of ROS and RNS, in fact, arise at low-moderate concentrations and involve physiological roles in cellular response to noxia (such as against infectious agents), in several cellular signaling pathways, and in the induction of a mitogenic response. Moreover, some ROS-mediated actions protect cells against ROS-induced oxidative damage and re-establish or maintain "redox balance". In contrast, an overproduction of ROS results in oxidative stress, with the involvement of oxidative stress in the ageing process having been well documented [6, 7].

Oxidative stress is defined as the disturbance in the balance between ROS/RNS, acting as oxidants, and levels of protecting antioxidants in favor of the former, potentially leading to damage [8], and is associated with a disruption of redox signalling and control [9]. According to the current state of knowledge, the pathological aggregation of cytoskeletal proteins and inactivation of key enzymes that occur in vulnerable regions of the brain in AD may be secondary to modifications induced by pro-oxidant substances [10].

The aim of the present work is to offer an overview of the current knowledge on oxidative stress as a pathophysi-

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ological step in AD that renders antioxidant intervention against AD a challenging but promising issue. The biological rationale for the use of antioxidants against cognitive impairment in AD, as well as the results of nutritional and antioxidant preclinical studies, will be discussed in detail.

2. BIOMARKERS OF OXIDATIVE STRESS IN ALZHEIMER'S DISEASE

The presence and relevance of a condition of oxidative damage in AD has been proven in a large body of cellular model and *in vivo* studies allowing the detection of byproducts of free radical- induced oxidative damage to DNA, RNA, lipids and proteins [11].

Increasing evidence supports a role for oxidative DNA damage in AD, as the attack of DNA by ROS, particularly hydroxyl radicals, can lead to strand breaks, DNA-DNA and DNA-protein cross-linking, and formation of at least twenty modified bases adducts. In addition, α- and β-unsaturated aldehydic byproducts of lipid peroxidation including 4-hydroxynonenal (HNE) and 2-propen-1-al(acrolein) can interact with DNA bases leading to the formation of bulky exocyclic adducts [12]. Modification of DNA bases by direct interaction with ROS or aldehydes can lead to mutations and altered protein synthesis. A significant increase of 8-hydroxy-2-deoxyguanosine (8-OHdG) in nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) isolated from three cortical areas and from cerebellum of AD patients was indeed found in comparison to controls, particularly in the parietal cortex [13], and in lymphocyte DNA from AD donors [14]. Increased 8-OHdG levels were also determined in ventricular cerebrospinal fluid (CSF) of AD patients [15]. Several studies of DNA base adducts in late-stage AD brain show elevations of 8-hydroxyguanine (8-OHG), 8-hydroxy-adenine (8-OHA), 5-hydroxycytosine (5-OHC), and 5-hydroxyuracil (5-OHU), a chemical degradation product of cytosine, in both nDNA and mtDNA isolated from vulnerable regions of AD brain compared to age-matched normal control subjects [for review see 16]. Using gas chromatography/mass spectrometry (GC/MS) with selective ion monitoring, it was found that levels of multiple oxidized bases (8-OHG, fapyguanine, 8-OHA, fapyadenine, 5-OHU and 5-OHC) in AD brain specimens were significantly higher in frontal, parietal, and temporal lobes compared to control subjects and that mtDNA had approximately 10-fold higher levels of oxidized bases than nDNA. Moreover, 8-OHG was approximately 10-fold higher than other oxidized base adducts in both AD and control subjects [17].

Measuring 8-OHG immunoreactivity, Nunomura and colleagues [18] found that oxidative damage to nucleic acids occurs predominantly in cytoplasmic RNA, rather than in nDNA, expressed by 8-OHdG, particularly in neurons within the hippocampus, subiculum, and entorhinal cortex as well as frontal, temporal, and occipital neocortex in cases of AD. Both immunoreactivities were increased as compared to control subjects. Recently the same author also found that patients carrying a presenilin-1 (PS-1) mutation, which results in a type of familial AD, show a considerable level of neuronal RNA oxidation [19]. Neuronal RNA oxidation is also a prominent feature of familial AD attributable to the amyloid β protein precursor (APP) gene, especially for those cases with a lower percentage area of Aβ42 burden [19]. Moreover, Shan and Lin [20] found that up to 30–70% of messenger RNAs (mRNA) are oxidized in AD frontal cortices. Identification of oxidized mRNA species revealed that some mRNAs are more susceptible to oxidative damage than others [23]; thus, RNA oxidation is not random but highly selective. Investigation of the consequence of oxidatively damaged mRNAs revealed that oxidized mRNA cannot be translated properly, leading to reduced protein expression, and loss of normal protein function. Finally, it was recently demonstrated that there is an elevation in RNA oxidation within the ribosome complex of Mild Cognitive Impairment (MCI) and AD, suggesting a role for RNA alterations within the ribosome as a mediator of decreased protein synthesis in both MCI and AD [22]. All these findings suggest that RNA oxidation is an early event involved in the pathological cascade of AD.

Lipid peroxidation, that causes structural membrane damage [23-26], has been prevalently assessed by measuring thiobarbituric acid-reactive substances (TBARS), malondialdehyde (MDA), isoprostanes, and HNE. Increased concentrations of TBARS were detected in AD frontal and temporal cortex compared with controls [27-30]. On the contrary, other studies failed to find significant differences in TBARS between AD and controls at basal condition, whereas TBARS concentration became higher in AD brains, after incubation with pro-oxidants [31, 32]. Despite TBARS have been criticized for their lack of specificity, sensitivity, and reproducibility, they remain one of the easiest and most frequently used parameters of lipid peroxidation [33]. Several studies reported no differences in MDA levels between AD brains and control brains [34-36], as well as no difference in lipid hydroperoxide levels [37, 38]. On the contrary, a selectively increased concentrations of F_2-isoprostanes, that have been directly demonstrated to accelerate Aβ generation and aggregation [39], were shown in brain areas characterized by the presence of Aβ deposition, NFT formation, and extensive neuronal death [40]. Higher total F_2-isoprostanes levels were determined also in CSF, urine and blood of demented patients [41, 42]. Several reports showed an increase in free HNE in multiple AD brain regions, including cerebellum, compared to controls [43], and particularly elevated in the amygdala and hippocampus [44]. It is worth of mention that HNE was recently found to covalently modify Aβ, triggering its aggregation into toxic oligomers [45]. HNE was found to inhibit the conversion of Aβ aggregates into straight fibrils and, on the other hand, enhance the misassembly of Aβ into small protofibrillar aggregates, that are strongly related to the pathogenesis of AD. HNE also induces the microtubule-associated protein tau to become resistant to dephosphorylation, which may contribute to neurofibrillary degeneration [46]. Finally, HNE inhibits neuritic outgrowth, disrupts neuronal microtubules, and modifies cellular tubulin, which may contribute to the cytoskeletal alterations that occur in AD [47, 48].

ROS and RNS also attack proteins, determining the formation of protein carbonyls and 3-nitrotyrosine. Protein carbonylations cause oxidation of side-chain, backbone fragmentation, formation of new reactive species (i.e. peroxides), discharge of other radicals, and occurrence of chain reaction [49]. Moreover, protein damage is irreparable and could lead to an extensive series of downstream functional conse-

quences, whereas several studies suggest that protein nitration could be a cellular signalling mechanism that is often a reversible and selective process [50, 51, for review see 52]. Nitration has been shown to alter protein function, including modification of catalytic activity, cell signalling, and cytoskeletal organization [53]. Protein carbonyls were significantly increased in both hippocampus and the inferior parietal lobule, but unchanged in the cerebellum, consistent with the regional pattern of histopathology in AD [54]. Increased levels of protein carbonyls were found in NFT as well as in the cytoplasm of tangle-free neurons [55, 56]. Increased 3-nitrotyrosine was found in both neurons containing NFT and in those in which they were absent [57, 58]. Again, the presence of nitrated tau in pretangles, tangles and tau inclusions in AD brain was recently demonstrated [59]. In this study, the expression of nitration was stronger in pretangles of early AD cases, compared to those of more advanced cases, suggesting that tau nitration may be an early event in AD. Increased levels of nitrated proteins, compared with those of controls, were found in the inferior parietal lobule and hippocampus of AD [60, 61]. Finally, several targets of protein nitration and carbonylation in AD brain have been identified by using redox proteomics [60-63]. Proteomics is a useful tool to identify specifically oxidized proteins in AD brain, because posttranslational oxidative modification of proteins in cerebral tissue may provide an effective means of screening a subset of proteins within the brain proteome able to reflect AD-related oxidative stress [64, 65]. Regarding peripheral markers of oxidative damage to proteins, a recent study showed that the degree of carbonylation of several CSF proteins did not vary between probable AD patients and controls [66], whereas 3-nitrotyrosine levels were reported to be elevated in the ventricular CSF of AD patients compared to controls [67, 68], and a significantly higher content of dityrosine in plasma immunoglobulin-G was demonstrated in AD patients as compared to controls [69].

Oxidized amino acids are rarely repaired and oxidized proteins are usually degraded by the 20S proteasome, a multicatalytic protease responsible for the majority of intracellular protein degradation, playing a pivotal role in several cellular activities including cell cycle regulation, antigen presentation and apoptosis [70-72]. Several studies have shown that, secondary to oxidative modification, the proteasome is impaired in AD, and can cause a reduction of the clearance of intracellular protein aggregates and degradation of oxidized and damaged proteins [73-75]. Among these, a recent study showed that remarkable decreases in proteasome-mediated protein degradation were observed in purified proteasome complexes from AD patients and also in MCI subjects suggesting that the loss of the proteasome-mediated protein degradation occurs at the earliest stage of AD [76]. Therefore, the accumulation of oxidized proteins in AD is likely a consequence of imbalance in any one of a number of different systems including free radical generation, antioxidant defences, efficiency of oxidized protein repair or removal.

3. BIOMARKERS OF OXIDATIVE STRESS IN MILD COGNITIVE IMPAIRMENT

Most of the studies reported above have been performed using cerebral tissue or peripheral samples from patients with moderate to severe AD. This approach does not determine whether oxidative damage is a late consequence of the disease or whether it occurs early in the disease process. The evidence for a role of oxidative stress also in the pathogenesis of MCI [77], which can be considered the very early phase of AD [78, 79], may be helpful to answer to this question.

Regarding DNA oxidation, 8-OHdG levels were found significantly elevated in nDNA and in mtDNA from frontal and temporal lobe of subjects with MCI compared to cognitively healthy controls [80]. Recent studies show elevated 8-OHG, 8-OHA, and 5,6-diamino-5-formamidopyrimidine in both nuclear and mtDNA isolated from vulnerable brain regions in amnestic MCI, suggesting that oxidative DNA damage is an early event in AD and is not merely a secondary phenomenon [16]. An increased DNA oxidative damage has also been also found in peripheral leukocytes of subjects with MCI compared to healthy controls [81]. Increased levels of 8-OHG as a marker of RNA oxidation were found in the inferior parietal lobule but not in the cerebellum of subjects with MCI [82].

One of the first studies of oxidative damage in the brain of subjects with MCI showed increased levels of TBARS and MDA in the temporal lobe, as compared to healthy controls [83]. Recently, it was also found elevated HNE levels in hippocampus, superior and middle temporal gyri, inferior parietal lobule and cerebellum, as well as elevated levels of acrolein in superior and middle temporal gyri [84-86], and significantly higher levels of F_2-isoprostanes in frontal, inferior parietal lobule, and occipital regions of subjects with MCI than in age-matched control followed longitudinally [87]. Increased levels of the isoprostanes 8,12-iso-$PF_{2\alpha}$-VI -a specific marker of *in vivo* lipid peroxidation [88] - were found to be significantly elevated in CSF, plasma and urine of subjects with MCI compared with controls [89], once more suggesting that lipid peroxidation may be an early event in the pathogenesis of AD. However, it is worth of mention the discrepancies otherwise observed between brain/CSF versus plasma/urine F_2-isoprostanes and F_4-neuroprostanes levels [90, 91]. A combined magnetic resonance imaging (MRI) and CSF marker study of living patients with MCI showed elevated level of CSF hyperphosphorylated tau and isoprostanes and decreased hippocampal volume in MCI subjects compared to controls [92]. Loss of phospholipid asymmetry in synaptosomes from the inferior parietal lobule of AD and MCI subjects, a signal for apoptosis, and elevated levels of apoptosis-related proteins recently were reported [93]. HNE or Aβ42 caused loss of synaptosomal lipid asymmetry [94].

Increased levels of protein carbonyls were detected in the superior and middle temporal gyri in subjects with MCI [83], and oxidation of several specific protein in the hippocampus of subjects with MCI were demonstrated using redox proteomics [95, 96].

Subjects with MCI showed also lower levels of non enzymatic antioxidants (vitamin A, vitamin C, vitamin E, uric acid and of the carotenoids lutein, zeaxanthin and β-carotene) and lower activities of enzymatic antioxidants (plasma and erythrocyte superoxide dismutase – SOD -, plasma glutathione peroxidase – GPx) as compared to cog-

nitively healthy controls [97]. Similarly, the plasma antioxidant status was shown to be significantly reduced in MCI subjects as compared to age-matched controls [98].

In conclusion, the evidence of oxidative damage against nucleic acids, lipids and proteins in subjects with MCI supports the hypothesis that oxidative damage is involved, as contributor, in the pathogenesis of neuronal degeneration in AD.

4. OXIDATIVE STRESS IN ALZHEIMER'S DISEASE PATHOGENESIS

A composite reciprocal relationship was shown between ROS production, Aβ deposition, tau protein, excitotoxicity and calcium dysregulation in the pathogenesis of AD [99-101]. Oxidizing conditions determine protein cross-linking and aggregation of Aβ peptides [102, 103], contribute to aggregation of tau [104] and other cytoskeletal proteins [105, 106]. On the other hand, Aβ aggregates provoke a succession of events that lead to the intracellular accumulation of ROS [107, 108]. Aβ may be also implicated in the oxidation of the nonsaturated carbohydrate, side chains of membrane lipids and in the disintegration of the neuronal membrane, that ultimately causes cell lyses [107]. Aβ peptide can damage neurons directly or indirectly through the activation of microglia [109-111]. Activated microglia can damage neurons by generating peroxynitrite ion ($ONOO^-$) via nitric oxide/superoxide (NO/O^-_2) pathways [112, 113]. The formation of elevated $ONOO^-$ concentrations can be the trigger for a cascade of events that may lead to oxidation of DNA, RNA, proteins, tyrosine nitration, enzyme inhibition, lipid peroxidation, and mitochondrial abnormalities [61, 96]. A product of the $NO/O^-_2/ONOO^-$ pathway can also trigger apoptosis, degeneration of neurons and dysfunction of the endothelial cells in the microvasculature of the brain [114, for review, see 115]. Further support for the association between oxidative stress and Aβ comes from a study which crossed mice with a knockout of one allele of a critical anti-oxidant enzyme, manganese SOD (MnSOD, SOD2) with a mouse that overexpressed a doubly mutated human APP (i.e., Tg19959), finding that the mice of the resulting cross demonstrated increases in both amyloid plaque burden and amyloid levels [116].

However, Aβ plays also many physiological and beneficial functions, some of which include redox-active metal sequestration [117], and SOD-like activity [118]. Moreover, Aβ has been shown to be inversely correlated with oxidative stress markers [119], suggesting that at very low concentrations it may have antioxidant effects [120]. Therefore, it was quite recently advanced the hypothesis that Aβ can be considered as a protective consequence to an underlying disease mechanism, and that the notorious lesions of AD represent a compensatory response [121, 122]. Consistent with this hypothesis, it was observed that oxidative damage decreases with disease progression, such that levels of markers of rapidly formed oxidative damage, which are initially elevated, decrease as the disease progresses to advanced AD. This finding indicates that ROS not only cause damage to cellular structures but also provoke cellular responses, such as the compensatory up-regulation of antioxidant enzymes found in vulnerable neurons in AD [123].

Evidence of an association between oxidative stress and tau comes from recent studies on animal models. Melov and colleagues [124] observed in *sod2* knockout mice a hyperphosphorylation of tau in most residues that has been previously associated with aggregated hyperphosphorylated tau in AD [125], and that this hyperphosphorylation was prevented with an increased dose of a catalytic antioxidant (EUK189). The authors concluded that hyperphosphorylated tau, which is caused by mitochondrial oxidative stress, could be resolved with appropriate antioxidant therapy. Dias-Santagata and colleagues [126], using *Drosophila* model of human tauopathies, that express a disease-related mutant form of human tau (tauR406W), showed that genetic and pharmacological up-regulation of antioxidant defences significantly rescues neurotoxicity in the brains of tau transgenic flies, whereas genetic inactivation of antioxidant mechanisms enhances tau-induced neurodegeneration. These observations strongly implicate oxidative stress as a critical mediator of tau-induced neurotoxicity.

5. THE BIOLOGICAL RATIONALE FOR THE USE OF ANTIOXIDANTS IN THE PREVENTION AND TREATMENT OF ALZHEIMER'S DISEASE

Antioxidants are widely discussed in both the lay press and the scientific literature as health-promoting agents that may protect against various age-related diseases. Nutritional and antioxidant micronutrient strategies against the development of AD are based upon the large body of evidence showing that antioxidant micronutrients can counteract the deleterious effects of oxidative stress in the organism, associated by nature with a higher risk for neurodegenerative diseases. As discussed above, there is sound rationale for hypothesizing that antioxidants could be prophylactic against central nervous system (CNS) diseases, especially in AD.

5.1. Antioxidant Molecules in Preclinical Studies

Preclinical animal studies are essential to understand disease and to build confidence in new therapeutic approaches; however it is becoming increasingly clear that scientists cannot extrapolate from non-human to human efficacy. At the same time, the scientific enterprise cannot accept the impossibility of mitigating CNS disease. A prudent middle ground would be to consider very judiciously the design and implementation of past animal studies that demonstrate antioxidant potential, with a goal of retesting these agents in experimental designs likely to better mimic a human clinical situation. Critical evaluation or re-evaluation would need to focus carefully on dosage, administration route and timing of drug treatment in such a way as to recreate not only the disease process but also to model a practicable human clinical trial design [127].

Preclinical and clinical interest in using antioxidants to treat AD stems from repeated observations that (1) oxidative stress is increased in AD brain beyond the levels of age-matched non-demented control human brain [5, 33, 54, 128, 129]; (2) amyloid peptides implicated in AD contribute to free radical-mediated processes [130-132]; and (3) oxidative damage can be produced in the brains of transgenic mice engineered to express a "Swedish" mutant amyloid precursor protein (APPsw) associated with a type of human familial AD [133].

The existence of murine models for amyloidopathy have allowed investigations into antioxidant therapeutics for AD with some success, although the long time-course of disease in these mice (twelve to eighteen months) and the need for cumbersome neurobehavioral evaluations have greatly inhibited progress in this area. Also these animals show only limited neuropathology and do not experience the profound frank neurodegeneration characteristic of human AD. Nonetheless substantial preclinical progress has been made, with certain caveats. Compounds with classical antioxidant (i.e., direct reducing or radical-scavenging ability) properties that have been shown to decrease amyloidopathy, mitigate behavioral decline or decrease brain protein oxidation in amyloid transgenic mice include vitamin E [134]; the curry spice curcumin [133]; the thiol compound pyruvate dehydrogenase/α-ketoglutarate dehydrogenase co-factor α-lipoic acid [135]; epigallocatechin-gallate esters from green tea [136]; and the tryptophan metabolite/neurotransmitter melatonin [137]. More recently other molecules with antioxidant properties such as tricyclodecan-9-yl-xanthogenate (D609) and ferulic acid ethyl ester (FAEE) have been tested with promising results.

In most cases, these agents reportedly diminish amyloid burden while simultaneously reducing protein carbonyls [133] or indices of lipid oxidation [134, 137]. Other studies have sought to test murine AD models against natural mixtures rich in putative antioxidants; among these include studies of apple juice [138] and even Cabernet Sauvignon [139]. Both of these latter interventions improved neuropathic variables in the experimental animals. Apple juice decreased expression of amyloid-processing PS-1 if apolipoprotein E (ApoE)-deficient mice were simultaneously deprived of dietary folate and vitamin E while the Cabernet promoted non-amyloidogenic processing in amyloid-overproducing Tg2576 mice.

These studies encourage further investigations of antioxidant agents for use against age-related dementias, but they do not constitute proof-of-concept for antioxidant therapeutics nor is there substantial reason to suspect the animal studies will extrapolate to the human disease, for the following reasons. First, none of these agents can be considered purely antioxidant and devoid of classical pharmacological modes-of-action. For instance curcumin inhibits amyloid aggregation *in vitro* [140] and possesses known pharmacological action upon eicosanoid-producing pro-inflammatory enzymes like cyclooxygenase (COX) and lipoxygenase [141]. Thus the reduction of brain protein carbonylation by curcumin might derive from classical anti-oxidant properties of the curcumin, or might represent a side-effect of the anti-inflammatory nature of the curcumin. Even if the former case is true (curcumin acting as an antioxidant), the reduction in carbonylation could be an independent epiphenomenon not relevant to disease pathology. This is a distinct possibility because vitamin E, the archetype for classical antioxidants, does not diminish brain oxidative damage in APPsw mice [142].

Setting aside issues regarding mechanisms-of-action, a pragmatic clinician immediately sees challenges to extending antioxidant benefits from the animal models to humans. Many agents used in preclinical AD studies, including curcumin, have extremely low bioavailability in humans. For instance, human volunteers consuming 3–8 g of curcumin per day do not achieve more than trace levels of circulating curcumin [143]. Likewise vitamin E is likely to load into adult brain very slowly and, in fact, only works in amyloidopathic mice when administration is begun at very early ages [134]. It is possible that formulations or encapsulations could be devised that would drive these molecules into the CNS of human patients newly diagnosed with, or in imminent risk for AD; but the human pharmacodynamic realities seriously undermine enthusiasm for use of these agents in human trials [143].

Vitamin E

Vitamin E is the most effective lipid-soluble antioxidant present in mammalian cells, and includes two groups of vitamins: tocopherols and tocotrienols [144]. In biological systems, α-tocopherol predominates and it is considered the most active form of vitamin E. Vitamins E has been shown to reduce Aβ-induced cell death in rat hippocampal cell cultures [145], and in cultured cortical nerve cells [146]. In addition, vitamin E suppresses brain lipid peroxidation, measured as 8,12-*iso*-$iPF_{2\alpha}$-VI levels, and significantly reduces Aβ levels and amyloid plaque deposition in a transgenic mice model of AD (Tg 2576), only when it is administered early during the evolution of their disease phenotype [134]. Further support for the association between vitamin E and Aβ comes from a recent study which crossed Tg 2576 mice with α-tocopherol transfer protein knockout ($Ttpa^{-/-}$) mice, founding that the double-mutant ($Ttpa^{-/-}$APPsw) mice showed earlier and more severe cognitive disfunction and increased Aβ deposits in the brain by immunohistochemical analysis, which was ameliorated with α-tocopherol supplementation [147].

Vitamin E may also prevent the oxidation of cysteine that contributes to the altered structure and function in the modified tau contained within NFT [148, 149]. Dietary deprivation of folate and vitamin E, coupled with iron as a pro-oxidant, fosters an increase in nonphospho- and-phospho-tau within brain tissue of mice homozygously lacking apolipoprotein E [138].

Curcumin

Curcumin (Fig. 1), has been shown to have antioxidant and antiinflammatory properties. It inhibits lipid peroxidation in rat liver microsome preparation [150], and prevents oxidative damage of DNA in mouse fibroblasts [151]. Furthermore, it has been demonstrated that curcumin inhibits lipoxygenase and COX-2, enzymes that are responsible for the synthesis of the pro-inflammatory leukotrienes, prostaglandins, and thromboxanes [152]. It also suppresses inducible nitric oxide synthase (iNOS) in activated macrophages [153], processes that promote inflammation. There are also evidences that curcumin has anti-amyloid properties, as it has been shown to inhibit the formation and extension of Aβ fibrils and destabilized preformed Aβ fibrils [140, 154].

The potential effect of curcumin were also studied in animal models of AD. Lim and colleagues [133], studying the effects of curcumin in APPsw transgenic mice, showed that low-dose of curcumin decreased total microglial activity

and decreased cerebral levels of oxidized proteins and of IL-1β, a cytokine that has been implicated in age-related memory loss [155]. Further, mice fed low-dose curcumin also had significantly reduced levels of soluble and insoluble Aβ as well as a reduced amyloid plaque burden.

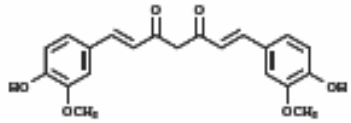

Fig. (1). Chemical structure of curcumin.

Lipoic Acid

Lipoic acid (Fig. 2), a naturally occurring precursor of an essential cofactor for mitochondrial enzymes, has a variety of properties which can interfere with pathogenic principles of AD. *In vitro* and *in vivo* studies suggest that lipoic acid acts as a powerful micronutrient with diverse pharmacologic and antioxidant properties: it has been shown to chelate redox-active transition metals, inhibiting the formation of hydrogen peroxide and hydroxyl radicals; scavenge intracellular ROS (acting as second messengers), increasing the level of reduced glutathione, and down-regulating inflammatory processes; scavenge lipid peroxidation products; and induce the enzymes of glutathione synthesis and other antioxidant protective enzymes [for review see 156 Holmquist *et al.*, 2007). Interestingly, in a *in vitro* experiment, Aβ from human AD brain and frontal cortex of APP overexpressing transgenic mice could be re-solubilized with transition metal ion chelators including lipoic acid [157], since amyloid aggregates have been shown to be stabilized by transition metals such as iron and copper [158, 159].

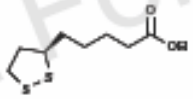

Fig. (2). Chemical structure of α-lipoic acid.

Lipoic acid, associated with acetyl-L-carnitine, was shown to play a protective role in cortical neuronal cells against HNE-mediated oxidative stress significantly attenuating HNE-induced cytotoxicity, protein oxidation, lipid peroxidation, and apoptosis in a dose-dependent manner [160].

The effect of lipoic acid, this time associated with and N-acetyl-cysteine (NAC), was also evaluated on oxidative and apoptotic markers in fibroblasts from patients with AD and age-matched and young controls. AD fibroblasts showed the highest levels of oxidative stress, and the antioxidants, lipoic acid and/or NAC exerted a protective effect as evidenced by decreases in oxidative stress and apoptotic markers. The protective effect of lipoic acid and NAC was more pronounced when both agents were present simultaneously [161].

Green Tea

The positive properties ascribed to tea consumption are believed to rely on its bioactive components, catechins and their derivatives (EGCG: (-)-epigallocatechin-3-gallate, (Fig. 3); EC:

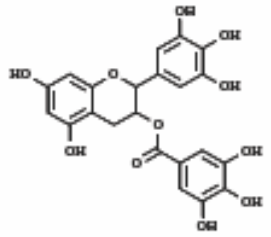

Fig. (3). Chemical structure of epigallocatechin gallate.

(-)-epicatechin; EGC: (-)-epigallocatechin; and ECG: (-)-epicatechin-3-gallate), which have been shown to act directly as radical scavengers and play indirect antioxidant effects through activation of transcription factors and antioxidant enzymes [for reviews see 162 Higdon and Frei, 2003; 163 Wiseman *et al.*, 1997). Tea polyphenols have been found to be potent scavengers of free radicals such as singlet oxygen, O^{-}_{2}, hydroxyl radicals, and peroxy radicals in a number of *in vitro* systems [164-166]. In the majority of these studies EGCG, the major green tea polyphenol component, was shown to be more efficient as a radical scavenger than its counterparts ECG, EC, and EGC. Moreover, *in vitro* observations show that EGCG, inhibits induced oxidative stress and neurotoxicity [167, 168], and EC reduces the formation of Aβ-fibril formation [169]. In addition, EGCG is able to regulate the proteolytic processing of APP both *in vivo* and *in vitro* [167].

Melatonin

Melatonin efficiently protects neuronal cells from Aβ-mediated toxicity via antioxidant and anti-amyloid properties [170]. It not only inhibits Aβ generation, but also arrests the formation of amyloid fibrils by a structure-dependent interaction with Aβ [171]. Recent studies have demonstrated that melatonin efficiently attenuates AD-like tau hyperphosphorylation [172]. Although the exact mechanism is still not fully understood, a direct regulatory influence of melatonin on the activities of protein kinases and protein phosphatases is proposed. Additionally, melatonin also plays a role in protecting cholinergic neurons and in anti-inflammation [173].

Tricyclodecan-9-yl-Xanthogenate (D609)

One of the antioxidants that is found in abundance in the brain is glutathione (GSH) [174]. In AD brain, the ratio of GSH and its oxidized form GSSG is altered as reported by reduced levels of GSH and increased levels of GSSG in specific regions that could contribute to the increased levels of oxidative stress that may eventually lead to neuronal cell dysfunction and neuronal loss [174, 175].

The endogenous elevation of GSH by i.p. injection of NAC or glutamylcysteine ethyl ester (GCEE) decreases the oxidative stress markers in synaptosomes and primary neuronal cultures treated with various oxidants including Aβ

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[176-180]. D609 (Fig. 4), is a xanthate molecule that mimics GSH functions. A disulfide is formed upon oxidation of D609 and the resulting dixanthate can then be catalytically reduced by GSH reductase similar to the reduction of GSSG as shown in a previous study [181, 182]. D609 given both *in vitro* and *ex vivo* has the ability to scavenge hydrogen peroxide and hydroxyl free radicals and can also bind to reactive alkenals and detoxify their effect, thereby preventing these alkenals from damaging mitochondria [183]. D609 also exhibits a variety of potent biological functions, including antiviral and anti-inflammatory activities.

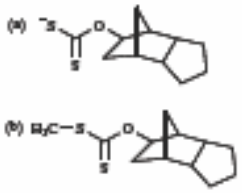

Fig. (4). Chemical structure of (a) D609, and (b) MD609.

D609 *in vitro* and *ex vivo* protected primary neuronal cells, mitochondria, and synaptosomes from Aβ(1–42)-induced oxidative stress as indexed by decreased protein oxidation and lipid peroxidation, decreased free radical formation, decreased 3-nitrotirosine formation, improved morphology and elevated mitochondrial function [183-186]. After pretreatment with Aβ(1-42) in primary neuronal cultures, three oxidatively damaged proteins involved in glucose metabolism, i.e., glyceraldehyde-3-phosphate dehydrogenase (GAPDH), pyruvate kinase, and malate dehydrogenase, and a chaperone protein, i.e., 14-3-3 zeta were identified. Among these proteins, two proteins, i.e., GAPDH and 14-3-3 zeta also are targets of oxidation in AD brain [61, 177, 187-189]. Pretreatment of neuronal cultures with D609 prior to Aβ(1-42) ameliorated the Aβ(1-42)-induced oxidative modification [189].

The decrease in oxidative stress by D609 can be related to the presence of a free thiol (–SH) group in D609 that may provide strong antioxidant activity with *in vitro* and *in vivo* radical scavenging properties and inhibition of free radical-induced oxidative stress [181, 182]. The critical role of the –SH group of D609 in protecting neurons against oxidative stress was solidified using the methylated form of D609 (MD609), which did not provide any significant protection against Aβ-induced oxidative damage or toxicity to neurons, suggesting the GSH mimetic D609 requires a free –SH functionality [185, 186]. Consistent with this notion, MD609 did not inhibit PC-PLC activity (Fig. 5). In addition, given that D609 increased the expression of stress responsive proteins such as heme oxygenase-1 (HO-1) and heat shock protein 72 (HSP 72) and decreased expression of iNOS [185], the protective nature of this xanthate could be related to these effects. Data so far obtained suggest that the neuroprotective effects of D609 involve multiple mechanisms such as its antioxidant property and also its ability to inhibit PC-PLC [181, 185, 186].

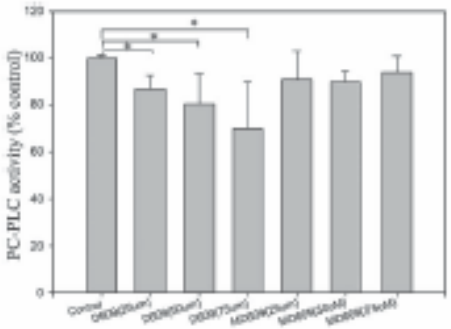

Fig. (5). Comparison of the effects of D609 and methylation of D609 (MD609) on PC-PLC activity in primary neuronal cell cultures.

Ferulic Acid Ethyl Ester (FAEE)

Phytochemicals are non-nutritive bioactive chemicals found in plants that can have beneficial effects on health [190-195]. Several studies performed to date have examined whether dietary intake of several phytochemicals, such as flavonoids, carotenoids, and vitamins, might prevent or reduce the progression of AD [196].

Ferulic acid (FA) is a ubiquitous plant constituent that arises from the metabolism of phenylalanine and tyrosine. FA occurs primarily in seeds and leaves both in its free form and covalently linked to lignin and other biopolymers. Due to its phenolic nucleus and an extended side chain conjugation, FA readily forms a resonance stabilized phenoxy radical which accounts for its potent antioxidant potential [197, 198]. Although FA has been demonstrated to be effective in *in vitro* experiments, the low lipophilicity impairs its *in vivo* efficiency, bioavailability, and stability. FAEE (Fig. 6), the naturally occurring ester derivative of ferulic acid, ranges widely within various systems of many plants as a trace constituent [199, 200]. In comparison with the corresponding acid form, FAAE is more lipophilic and has been shown to present better scavenging properties toward both hydroxyl radicals and O^{-}_{2} [198, 201-203]. In addition to the radical-scavenging activity of an antioxidant, both its polarity and its three-dimensional interaction with lipid bilayers may contribute to its antioxidant activity. Synaptic membranes are particularly vulnerable to oxidative stress, so the ability of an

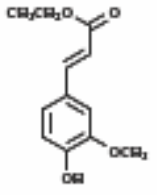

Fig. (6). Chemical structure of FAEE.

antioxidant to act at membrane sites because of its high lipophilicity results in a higher antioxidant potential. The synapse is one of the primary targets of Aβ-mediated neurotoxicity, so the affinity of FAEE with lipid substrates might be an important factor in modulating Aβ-induced oxidative damage.

Considering the increased lipophilicity of FAEE, in *in vitro* or *ex vivo* studies showed decreased protein oxidation and lipid peroxidation, decreased free radical formation, decreased 3-nitrotirosine formation, improved morphology and elevated mitochondrial function in primary neuronal cultures treated with Aβ(1-42) and on synaptosomes isolated from FAEE-treated rodents and subsequently treated with Aβ(1-42) or with Fe^{2+}/H_2O_2 (hydroxyl radical formation), or with AAPH (alkoxyl and peroxyl radical formation) [204, 205]. The mechanistic basis of the neuroprotective activity of FAEE appears to rely not only on its general free radical trapping or antioxidant activity *per se*, but also on its activity in mediating the induction of stress response proteins (HO-1 and HSP72) and the parallel suppression of genes induced by pro-inflammatory cytokines, such as iNOS. FAEE-treated cells may strengthen the cellular defense mechanism similar to the effects of curcumin [206].

In summary, the preclinical studies of antioxidant pharmaceuticals in AD models suffer from several shortcomings. In general the studies require extreme and multi-factorial manipulations of mice to produce a model somewhat reminiscent of human AD; and equally heroic manipulations of diet in order to partially mitigate the observed CNS pathology. The studies often do not attempt to evaluate active agents in animal models within a pharmacological concentration range that could be reasonably achieved in humans, nor are studies generally undertaken with the goal of slowing disease when drug administration is begun late in life as would be the case in a human clinical trial. The studies do not always attempt to test whether or not the palliative actions of the test agent arise from the agent's antioxidant properties or other, pharmacological and possible oxidation – independent modes of action. Studies involving crude mixtures of agents are particularly problematic and generally cannot be interpreted because several variables are changed simultaneously without cross-controlling for effects of differing caloric content; taste novelty; micronutrient enrichment and peripheral (extra-CNS) effects that would not be germane to human clinical physiology. Finally, when efforts are made to measure indices of oxidative stress, the studies often do not measure multiple indices and frequently rely on nonspecific biomarkers such as TBARS.

5.2. Dietary Antioxidants and Antioxidant Supplementation in Humans

Basically, there are two approaches for antioxidant implementation and supplementation in humans, i.e. the use of antioxidant-rich foods in the diet and the use of antioxidant supplements.

The human diet provides both macronutrients, which are the main source of calories, and micronutrients (roughly forty essential minerals, vitamins, and other relevant compounds), which are required for specific metabolic processes. Antioxidant micronutrients and vitamins scavenge free radicals and other reactive species thereby providing protection against oxidative reactions and oxidative stress. Small molecules with antioxidant properties such as ascorbate (vitamin C), tocopherols (vitamin E) and carotenoids are provided to the organism through the diet – particularly through the intake of fruits and vegetables – and are therefore called antioxidant micronutrients. They are an essential component of the antioxidant defense network, and an inadequate supply of these compounds with the diet has been epidemiologically correlated with an increased risk for degenerative diseases. As the main dietary sources of energy in the developed countries are rich in carbohydrates and fats but poor in micronutrients, large portions of the population are subjected to micronutrient malnutrition [207] and individuals undergo a higher risk for the development of what has been previously called 'hidden hunger'. Although genetic variation has been also shown to significantly influence human nutritional requirements [208], the elderly and the obese are the individuals mostly at risk for micronutrient malnutrition. Older adults are generally at greater risk for nutritional deficiencies than are younger adults. Among seniors, food choice and related activities are affected by health status, biological changes influenced by aging and functional abilities, which in turn are mediated by familial, social and economic factors. Determinants of healthy eating stem from individual components, such as age, gender, education, physical and psychological attributes, lifestyle, behavior, income, social status and culture, as well as from collective factors including accessible food labels, shopping and marketing environment, adequate social support and the availability of community-based meal delivery services. In the elderly, the decrease in energy requirement due to a reduction in lean body mass is associated to a decrease in macro- and micronutrient intake of 30% by the age of 80 years [209]. A reduction in energy expenditure is also associated with sedentary behavior and a loss of mobility related to systemic (e.g. cardiovascular and pulmonary) or bone and joint disease. To avoid weight gain, which may synergistically add to already existing functional deficits, dietary intake may be decreased. A reduction in nutrient consumption, however, is often achieved through a decrease of the overall dietary quality due to the intake of favorite foods in spite of those that are less well-liked or more difficult to prepare (fruits and vegetables). Nutrient-dense foods often fall into the category 'unliked', while favorite foods are rich in calories and high in carbohydrates and fats [210]. Risk for poor nutritional status is also related to a decreased efficiency of the gastrointestinal tract that occurs in some elderly people. Chewing, swallowing, digesting, and absorbing nutrients may be impaired for a variety of reasons.

As advanced age is threatened by the inadequacy of daily diets, not obtaining sufficient amounts of micronutrients becomes a challenge within the complex nature of the human aging process, and dietary references intakes have been established for older adults [http://nutritionandaging.fiu.edu]. In order to guarantee an adequate antioxidant micronutrient status, physicians, nutritionists and dieticians encourage the consumption of at least five portions of fruits and vegetable per day. Unfortunately, only less than one fourth of the population in developed countries consumes the suggested amounts of fruits and vegetables. Inadequate nutritional in-

take is rampant among the elderly, affecting nearly 44% of otherwise healthy, community-dwellers and up to 61% of aged patients in developed countries [211, 212]. Nutrient intakes in a rural, community-dwelling elderly population were shown to be inadequate for folate, vitamin D, vitamin E, calcium and magnesium in over 60% of the population studied whereas one-fourth of the subjects did not meet their estimated needs for vitamin B_{12}, B_6, vitamin C and zinc [213]. Malnutrition, as one of the most common comorbidities in the elderly, may exacerbate cardiovascular and cerebral subclinical disturbances associated with aging, affecting vascular disease and neurodegenerative disease outcomes as well as longevity.

The suboptimal consumption of micronutrient-rich food like fruits and vegetables and a micronutrient intake below the Recommended Dietary Allowance (RDA) but still above the level causing typical vitamin deficiencies keeps the elderly at constant risk of disease development. Among micronutrients, low intakes and circulating levels of antioxidants are in turn associated with an increased load of ROS, RNS and chlorine (reactive chlorine species, RCS).

The antioxidant defense system is indeed organized at the levels of prevention, interception and repair. Prevention comprises strategies to avoid the generation of ROS/RNS/RCS, whereas repair is mediated by enzymes which recognize oxidatively damaged molecules and initiate their repair, degradation or removal. A network of antioxidant enzymes is available for the interception of ROS/RNS/RCS once they are generated, including catalase (Cat), GPx and SOD. Since a decrease in the activity of any of these antioxidant enzymes was found to cause cellular death [214, 215], an abnormality in the activity of any one of these three enzymes may determine the excessive neuronal cell death found in AD [30]. Furthermore, studies on plasma and erythrocyte antioxidant enzymes have largely found decreased activities in AD patients compared to controls [for review, see 7].

The assessment of circulating antioxidant micronutrients can be used for several, equally important purposes. First of all, together with other information, e.g. biomarkers of ROS-mediated damage, plasma or serum micronutrient evaluation serves to address presence and entity of conditions of oxidative stress [216]. Secondly, it helps identifying a state of micronutrient inadequacies [216]. A third advantage of an accurate and reliable micronutrient measurement is the possibility of exploring micronutrient changes, behavior or simply status in relation to a reliable clinical indicator simultaneously measured in a given disease. This latter approach, unfortunately rarely used in clinical studies, might help recognizing early pathophysiological mechanisms and appropriate intervention timing in age-related diseases with micronutrient malnutrition. The use of the measurement of micronutrient networks in identifying a condition of hidden malnutrition clearly displays in AD, in which a broad spectrum of plasma antioxidant micronutrients and vitamins are significantly decreased compared to controls [for review, see 7] and appear to be inversely related to the lymphocyte DNA content of 8-OHdG [217].

As far as dietary intervention in disease prevention is concerned, it is now widely believed that the actions of the antioxidant nutrients alone do not explain the observed health benefits of diets rich in fruits and vegetables because, taken alone, the individual antioxidants studied in clinical trials do not appear to have consistent preventive effects. Fruits and vegetables are thought to represent the best source of antioxidant micronutrients due to synergisms of their components, because they may allow a better bioavailability of protective compounds than single vitamins, and due to their low content in saturated fats. The preventive effect against chronic degenerative diseases, in fact, is much stronger and consistent for antioxidant-rich fruits and vegetables than for single compounds [218-225].

A condition of malnutrition has been shown to be associated with a more rapid worsening of AD [226]. With the exception of one study [227], an association between high dietary antioxidant intake and a decreased risk for AD has been consistently reported [for review, see 228]. However, intervention trials demonstrated no major benefit with antioxidant supplementation in the treatment of AD [229]. It is possible that when the clinical symptoms of AD appear, a large proportion of neuronal cells might already be destroyed and therefore the intervention with antioxidants, especially when a single compound is used instead of a micronutrient network, could come too late. In order to evaluate whether the often-reported age-related decrease of plasma antioxidants in humans depends on differences in dietary intake or on other age- and gender-related factors, elderly community-dwelling healthy subjects were studied, a group consuming high intakes of fruits and vegetables daily (HI group, $\geq$ 4 portions/day, > 350 g/day), the other with low intakes of fruits and vegetables daily (LI group, 0-1 portions/day, 0-100 g/day). Antioxidant micronutrients including vitamins A, C, E and six carotenoids were measured in plasma by means of HPLC. The observation that higher antioxidant micronutrient plasma concentrations and lower levels of oxidative stress biomarkers in healthy elderly subjects in the HI group compared to the LI group suggests that diet plays indeed a crucial role in determining the favorable oxidant/antioxidant balance of the organism also in advanced age [230]. Recently, the effects of dietary counseling on fruit and vegetable intake as well as of improved fruit and vegetable intake on the levels of circulating antioxidants and biomarkers of oxidative stress was studied. One-hundred twenty-nine employees of a University hospital followed a diet consisting of at least five portions of fruits and vegetables per day over three months. Fruit and vegetable intake was monitored, counseling sessions were offered and blood samples were obtained. Several antioxidants were measured over the course of the study along with biomarkers of lipid peroxidation and protein oxidation. A significant increase in several plasma antioxidant micronutrients in the absence of changes of biomarkers of oxidative stress during the course of the study in this health-conscious study population (mostly females, relatively young, well educated) was observed [Polidori *et al.*, unpublished data]. These results suggest that a nutritional counseling program can lead to improvement in plasma antioxidant status even in a health conscious population of professionals, in which a relevant decrease in biomarkers of oxidative stress is not to be expected. In patients with a typical age-related disease such as AD, showing increased circulating levels of biomarkers of oxidative stress, a

targeted nutritional intervention aimed at increasing plasma antioxidant levels and at decreasing the ongoing condition of oxidative stress might prove beneficial in addition to standard therapeutic options. In a prospective cohort study of over 3,700 older participants of the Chicago Health and Age Project, high vegetable consumption was associated with a slower rate of cognitive decline over six years after adjusting for age, gender, race, education, cardiovascular-related conditions and risk factors [231]. In this study the consumption of green leafy vegetables, rich in antioxidant micronutrients like carotenoids, showed the strongest inverse linear association with the rate of cognitive decline. The specific protection shown by vegetables and particularly by the green leafy ones appears to be in disagreement with the concept that fruit and vegetable consumption might be beneficial in the frame of a generally healthy lifestyle, as health-conscious individuals tend to consume both fruits and vegetables.

Finally, epidemiological studies indicated that moderate red wine intake reduces the risk of developing AD [190-192]. Resveratrol, a polyphenol that occurs in abundance in red grapes and is found in red wine, exerts antioxidant and neuroprotective properties and therefore contributes to the potential beneficial effect of wine consumption on the neurodegenerative process [193, 194]. Marambaud *et al.* [195] reported that resveratrol has a potent anti-amyloidogenic activity by reducing the levels of Aβ produced from different cell lines expressing wild type or Swedish mutant APP_{695}.

5.3. Antioxidants in Clinical Trials

Increased production of free radicals in the AD brain are likely an essential factor in the pathogenesis of AD and, consequently, are a potential target of therapeutic strategies. As such, therapeutic modalities encompassing antioxidants may be an effective approach to the treatment of neurodegenerative diseases.

Vitamin E and selegiline (a monoamine oxidase B inhibitor), were investigated as part of the Alzheimer's Disease Cooperative Study in patients with moderately severe AD [232]. A controlled clinical trial with α-tocopherol (synthetic form: 2000 IU/day) in patients with moderately severe impairment from AD showed some beneficial effects with respect to rate of deterioration of cognitive function [232]. In the same α-tocopherol clinical trial, selegiline (10 mg/day) produced beneficial effects similar to that produced by α-tocopherol. Although any significant improvement on cognitive tests was observed, significant delays in the time of the occurrence of severe dementia and death were otherwise observed. These findings provided supporting evidence for the hypothesis that antioxidants may be capable of slowing the pathogenic process in AD. Interestingly, there was no significant difference in effect between the groups receiving a combination of α-tocopherol and selegiline and those receiving treatment with the individual agent [232, 233]. Several possibilities were proposed to explain the lack of additive effect. One of them was that selegiline and vitamin E may act by the same mechanism. Indeed, both reduce the levels of free radicals, although by different mechanisms. Vitamin E, a chain-breaking antioxidant, protects neurons by "quenching" formed ROS, whereas selegiline protects neurons by preventing the formation of ROS and by inhibiting oxidative metabolism of catecholamines. Therefore, clinical studies involving vitamin E and selegiline support the concept that ROS are one of the intermediary risk factors for the progression of neurodegeneration in AD [234]. However, in a randomized clinical trial of three years of observation, vitamin E (2000 UI daily) failed to prevent progression of MCI to dementia: during the three years of the trial, in fact, there were no significant differences in the probability of progression from amnestic MCI to AD on the basis of the Cox analysis between the vitamin E group (n. 257) and the placebo group (n. 259) (HR 1.02; 95% CI: 0.74-1.41; p=0.91), and there were few significant differences in cognitive function, that represent the secondary outcome measures, from baseline between the vitamin E and placebo groups [235].

The therapeutical use of l-acetyl-carnitine in AD has not been supported by randomized clinical trials. Two open-label studies on small populations of patients with moderate AD showed a reduced progression of the disease, particularly in the cognitive performances, but further, better designed studies are needed [236, 237].

Recent studies showed that melatonin may play an important role in aging and AD as an antioxidant and neuroprotector [173, 238]. Melatonin decreases during aging and patients with AD have a more profound reduction in this hormone. The only randomized double blind clinical trial with melatonin for treatment of sleep disturbances in patients with AD did not show any significant benefit [239].

In a 2001 relatively small study (n. 47 randomized subjects), the glutathione elevating agent NAC failed to achieve formal significant differences in overall disease progression across a 6-month trial period, evaluated by a test of general cognition (Mini Mental State Examination, MMSE, and by scale that assessed basic and instrumental activities of daily living; however comparison of interval change from 3 to 6 months favored NAC treatment on MMSE score and on other test that evaluated language and memory function [240].

The studies performed on Ginko Biloba extract EGb 761 showed positive effects on cognitive indexes, similar to the results obtained with tetrahydroaminoacridine [241]. Conversely, neither α-tocopherol nor selegiline showed any significant effect on cognition. The main effects of Ginkgo biloba extract in the central nervous system seem to be related to its antioxidant properties, which require the synergistic action of flavonoids, terpenoids and organic acids. These compounds, to varying degrees, act as scavengers for free radicals, which have been considered mediators of excessive lipid peroxidation and cell damage observed in AD [242]. However, it is interesting to note that EGb 761 has many other effects in addition to its free radical–scavenging activity, including a protective effect on neuroreceptors in aging subjects, a monoamine oxidase–inhibiting effect (less than selegiline), and a clear effect on cognitive indexes [243].

Nonsteroidal antiinflammatory drugs (NSAIDS) have also been proposed as having a useful effect in countering AD [244, 245]. These drugs operate principally by acting on cyclooxygenases and inhibiting prostaglandin synthesis, or by decreasing glutamate-related excitotoxicity and reducing the production of ROS. However, clinical trials of NSAIDS in AD were disappointing [246-253].

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Accumulated evidence from basic science and clinical research studies suggests that estrogen could play a neuroprotective role [254-256]. Its beneficial effects on the brain include regulation of synaptogenesis and neurotransmitter and hormone levels, inhibition of the inflammatory process, antioxidant activity, augmentation of cerebral blood flow, and prevention of apoptosis [257]. Conjugated estrogens delivered orally to ovariectomized rats appeared to inhibit both cerebrovascular Abeta toxicity and damage related to Aβ peptide activity. Behl *et al.* [258] showed that 17β-estradiol protects neuroblastoma cells against oxidative stress, and Goodman *et al.* [259] showed that 17β-estradiol and estriol suppress membrane oxidation in hippocampal neurons induced by Aβ [258]. In addition, Fillit *et al.* [260] described a cognitive improvement in patients with AD who received estrogen treatment for 6 weeks. Since that study, a growing body of literature has shown that estrogen therapy may contribute to the prevention, attenuation, or even the delay of the onset of AD [261, 262]. Furthermore, estrogen replacement may also facilitate other treatments used for AD. For example, in clinical trials that used tacrine, an anticholinesterase drug, a greater efficacy was seen in women receiving estrogen therapy than in women who were not [263]. However, more recent studies have seriously questioned both the efficacy and safety of estrogen as a long-term therapeutic strategy for neurodegeneration [264].

CONCLUSIONS

A review of studies conducted over the past twenty years, that tested agents claimed to be "antioxidant" in animal models of AD with strongly-implicated oxidative stress components, revealed a generally favorable series of outcomes, with the antioxidant therapies proving largely efficacious across the models. In contrast, a review of the literature regarding antioxidant efficacy in human supplementation trials or clinical drug trials revealed little to no effect of commonly-employed antioxidant substances. Even more disconcerting, there is emerging data that suggests very high-dose or long-term supplementation with vitamin E may pose certain health risks.

The reasons for the disjunction between the animal model studies and the human clinical experience may be due either to a flaw in the theory concerning oxidative stress as a pathological contributor to neurodiseases; or a flaw in the implementation of that theory. Theory would be flawed if oxidative stress plays no role in neuron dysfunction or death; which is to say, if oxidative stress is merely an epiphenomenon. In this case antioxidant interdiction would likely decrease biomarkers of oxidative stress in animal models (or humans) without imparting an observable benefit on neuron viability, histological indicators or behavioral outcomes. Evidence from animal models suggests this is not the case; generally speaking, purported anti-oxidant therapies both diminish oxidative damage (e.g. measured by protein carbonylation in the case of curcumin in murine models of AD) and also slow disease progression. The caveat in this last statement is that most studies of putative antioxidants do not simultaneously report oxidative stress measures and pathological endpoints so it is difficult to correlate the diminution of oxidative stress biomarkers with the overall phenotypic / outcomes benefit of the test agents. This is an area that can be improved in future studies, especially with the advent of increasing numbers of technically facile assays for monitoring oxidative stress in tissue lysates.

Likewise, there is abundant evidence that oxidative stress occurs in AD pathogenesis; however there is little experimental data from human studies to discern whether this exacerbation of oxidative stress is contributive to the severity of the disease, because human studies necessarily must be observational in nature. Nonetheless it would appear that many animal models do recapitulate the oxidative stress component of their corresponding human disease counterpart.

If antioxidant theory is valid, then the implementation of antioxidant strategy must be flawed. There is abundant reason to suspect this is the case. Academic studies generally are performed in such a way as to bias in favor of a treatment effect, by administering large concentrations of test agent early in disease or before experimental disease or injury occurs. This may be appropriate in early studies to prove a concept, but such studies do not in any way mimic the human clinical situation. In order for a preclinical study to engender confidence that a test agent might work in a human clinical situation, the test agent would need to impart benefit to the non-human model at dosages and times that might be achieved safely and practically in a human. Of course accomplishing such objective would be very difficult as it would require routine pharmacokinetic assessments of drug disposition in the animal model, and ideally in comparison with known human pharmacological parameters. Nonetheless, animal studies will continue to fail in their prediction of human clinical efficacy unless more attention is devoted to "humanizing" the animal research.

Finally the oxidative stress and antioxidant research community needs to attend to the meaning of the term "antioxidant". The term is applied loosely to mean any agent that decreases oxidant concentration by either scavenging oxidants catalytically (e.g., the metalloporphyrins) or stoichiometrically (as in the case of nitrone-based free radical traps). In reality however, many substances can be antioxidant *in vivo* under conditions that are not relevant *in vivo*. More to the point, many antioxidants actually contain inherent pharmacological activity by virtue of binding to and reacting with specific receptors or enzyme targets. For instance, curcumin acts as chain-breaking antioxidants *in vitro* but also bind and antagonize cyclooxygenase and lipoxygenase, at nanomolar concentrations. The pharmacological activity of such compounds may result in the diminution of oxidant production and hence be a very potent and true "antioxidant" mode of action *in vivo*; however, studies of such multifunctional compounds should consider the extent to which benefits in animal models arise from drug-induced changes in paracrine signaling dynamics, such as circuits driven by eicosanoids and prostacyclins.

One might argue that the mechanism of action of an antioxidant is not germane to estimating the likelihood that the agent's efficacy will extrapolate from preclinical studies to human clinical trials. On the contrary, an understanding of a drug's mechanism of action is crucial to the design and implementation of human trials, for example to avoid undesirable activity of the drug at target sites outside the diseased

organ or to avoid dosage at inappropriate times when the target-of-action is temporally pleiotropic (e.g., beneficial early in the course of disease but detrimental later in the disease).

The success of multitudinous preclinical antioxidant studies overwhelmingly obligates the scientific community to continue researching oxidative stress with long-term goals of manipulating oxidative stress processes for clinical benefit. Despite the recent clinical disappointments of antioxidant therapies, sound rationale remains to develop improved antioxidant pharmacophores or formulations for the prophylaxis and mitigation of human neurodegenerative disease.

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LIST OF ABBREVIATIONS

Aβ	=	β-Amyloid
AD	=	Alzheimer's disease
ApoE	=	Apolipoprotein E
APP	=	Amyloid β protein precursor
APPsw	=	Swedish mutant amyloid precursor protein
Cat	=	Catalase
CNS	=	Central nervous system
COX	=	Cyclooxygenase
CSF	=	Cerebrospinal fluid
D609	=	Tricyclodecan-9-yl-xanthogenate
mtDNA	=	Mitochondrial DNA
nDNA	=	Nuclear DNA
EC	=	(-)-Epicatechin
EGC	=	(-)-Epigallocatechin
EGCG	=	(-)-Epigallocatechin-3-gallate
FAEE	=	Ferulic acid ethyl ester
GAPDH	=	Glyceraldehyde-3-phosphate dehydrogenase
GCEE	=	Glutamylcysteine ethyl ester
GC/MS	=	Gas chromatographymass spectrometry
GPx	=	Glutathione peroxidase
GSH	=	Glutathione
GSSG	=	Oxidized glutathione
HNE	=	4-Hydroxynonenal
HSP 72	=	Heat shock protein 72
MCI	=	Mild Cognitive Impairment
MDA	=	Malondialdehyde
MMSE	=	Mini Mental State Examination
MRI	=	Magnetic resonance imaging
NAC	=	N-acetyl cysteine
NFT	=	Neurofibrillary tangles
NO	=	Nitric oxide
iNOS	=	Inducible nitric oxide synthase
NSAID	=	Nonsteroidal antiinflammatory drug
$O^{\cdot}_2$	=	Superoxide
8-OHA	=	8-Hydroxyadenine
5-OHC	=	5-Hydroxycytosine
8-OHdG	=	8-Hydroxy-2-deoxyguanosine
8-OHG	=	8-Hydroxyguanine
5-OHU	=	5-Hydroxyuracil
$ONOO^-$	=	Peroxynitrite ion
PS-1	=	Presenilin-1
RCS	=	Reactive chlorine species
RDA	=	Recommended dietary allowance
mRNA	=	Messenger RNA
RNS	=	Reactive nitrogen species
ROS	=	Reactive oxygen species
SOD	=	Superoxide dismutase
SOD2	=	Manganese superoxide dismutase
sod2	=	Manganese superoxide dismutase gene
TBARS	=	Thiobarbituric acid-reactive substances
$Ttpa^{-/-}$	=	α-Tocopherol transfer protein

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